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14. ABSTRACT

Foxp3, the first identified X-linked prostate tumor suppressor, represses c-Myc in both the mouse and human prostate. Dysfunction of the Foxp3-c-Myc axis may lead to prostate cancer initiation. Tsc1 and Foxp3 double-deletions in the mouse prostate led to prostate carcinoma at an early age. In this proposed study, we observed that deletion of Tsc1 led to a constitutive mTOR activation and subsequently increases phosphorylation of c-Myc at threonine 58 (pT58) and decreases phosphorylation at serine 62 (pS62). Furthermore, loss of Foxp3 transcriptionally induces c-Myc expression and loss of Tsc1 activates mTOR signaling, leading a cross-talk between Foxp3-c-Myc and Tsc1-mTOR signaling pathways that converges on c-Myc and promoted tumor progression. This observation will help us understand how double Foxp3 and Tsc1 deficiencies promote tumor progression of prostate cancer.

15. SUBJECT TERMS
Prostate cancer, Tumor progression, Gene therapy, Transcriptional regulation, Post-translational modification

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Table of Contents

INTRODUCTION

Human prostate cancer is thought to be a heterogeneous population of mutant cells caused by the accumulation of mutations in tumor-related genes within multiple signaling pathways. These accumulated mutations may cause the transformation of benign prostatic epithelium to prostatic intraepithelial neoplasia (PIN), with progression to invasive carcinoma and, ultimately, metastatic disease [1-3]. Mouse models of PIN (mPIN) generated by a single-mutant gene in prostate do not progress to invasive carcinoma [1, 2]. However, overexpression of c-*Myc* or conditional deletion of *Pten* in mouse prostate epithelium most accurately replicates the development of human prostate cancer [4, 5], suggesting that the c-Myc- or Pten-signaling pathways have a critical role in prostate tumor progression.

FOXP3 at Xp11.23 is a member of the forkhead-box/winged-helix transcription factor family. This gene functions as the master regulator in the development and function of regulatory T cells [6]. However, *FOXP3* is also expressed in epithelial tissues of the breast, lung, and prostate [7]. Nuclear FOXP3 is observed in normal human prostate epithelial cells but is lost in approximately 70% of human prostate cancers [8]. In prostate cancer samples, *FOXP3* is frequently inactivated by deletion (14%) or somatic mutation (25%) by a single-hit inactivation mechanism [8]. *FOXP3* mutations reported in prostate cancer cells abrogate normal FOXP3 function by disrupting its nuclear translocation [8]. Moreover, an inverse correlation exists between *FOXP3* and *c-MYC* expression in human primary prostate cancers [8]. *FOXP3*-mediated transcriptional repression of c-*MYC* is necessary to control c-MYC levels in normal prostate epithelial cells [9]. Indeed, inactivation of FOXP3 contributes to the widespread overexpression of *c-MYC* in prostate cancer [8] while ectopic expression of wild-type (WT) *FOXP3* induces substantial growth inhibition of prostate cancer cell lines [8]. Lineage-specific ablation of *Foxp3* in mouse prostate epithelial cells leads to mPIN, as well as a dramatic increase in both c-*Myc* mRNA and protein expression, further suggesting that Foxp3 is a critical repressor for the c-*Myc* locus [8]. These data indicate that FOXP3 is an X-linked tumor suppressor in both the mouse and human prostate, and suggest that genetic alteration of *FOXP3* is an early event in prostate carcinogenesis.

The PI3K/Akt/Tsc1/2/mTOR signaling pathway is upregulated in 30–50% of prostate cancers, often through loss of PTEN suppressor function [10, 11]. Recent studies that systematically analyzed large cohorts of human prostate cancer patients to identify mutated genes in aggressive and metastatic prostate cancer confirmed that the most frequently mutated genes are *PIK3CA* (4% mutation and 15–20% amplification) and *PTEN* (4% mutation and 30–39% deletion) [12, 13]. Notably, the mutated or deleted genes identified in prostate cancer cells lead to constitutive activation of PI3K/AKT/mTOR signaling [13, 14]. Mice heterozygous for *Pten* deletion develop mPIN with 100% incidence, and homozygous deletion of *Pten* in the prostate induces aggressive prostate cancer [5, 15, 16]. Mutant *Akt1* transgenic mice develop mPIN with pathologic similarity to mPIN in *Pten*$^{+/-}$ mice [17]. Ultimately, PI3K/Akt signaling activates mTOR, and enhanced levels of mTOR and its downstream targets are observed in prostate cancer [18]. Importantly, genetic loss of *Akt1*, *mTOR,* or *eIF4E* (mTOR downstream target) is sufficient to significantly reduce the initiation of prostate cancer in the *Pten* conditional knockout mouse model [19-21]. Overall, these genetic studies demonstrate the importance of the PI3K/Akt/mTOR pathway in prostate cancer development and progression. Tsc1 is an essential component of the mTOR pathway. Either phosphorylation of the Tsc1/2 complex by Akt or loss of Tsc1/2 facilitates mTOR activation [22, 23]. *Tsc1*-deficient mice develop mPIN in the lateral

and anterior prostate by 27 weeks of age, with increasing disease penetrance over time [24], and lateral prostate lesions progress to prostate carcinoma in 16- to 22-month old mutant mice [24]. Thus, release of Tsc1-dependent mTOR inhibition is sufficient to initiate prostate tumor progression. While genetic alterations in *TSC1/2* have not been reported previously in human prostate cancers, a missense mutation (G1034C) of *TSC1* was identified in the prostate cancer cell line DU145 (www.mskcc.org).

While there are no reports citing a direct connection between Tsc1 and Foxp3 or c-Myc, the role of Tsc1 opposes that of c-Myc in cell growth and proliferation [25-27]. c-Myc activation appears to correlate with PI3K/Akt/mTOR signaling in prostate cancer [28], but the underlying molecular mechanisms remain unknown. However, three potential possibilities exist. 1) mTOR directly regulates c-Myc stability: Expression of c-Myc is affected by phosphorylation at the conserved residues threonine 58 (T58) and serine 62 (S62). Phosphorylation at S62 (pS62) stabilizes c-Myc, while phosphorylation at T58 (pT58) promotes c-Myc ubiquitylation and is required for its degradation [29-31]. Results from recent studies suggest that c-Myc is regulated by proteins identified in phosphorylation peptide libraries of mTOR complex 1 (mTORC1), one of the complexes through which mTOR functions [32, 33]. Thus, mTOR activity may affect the stability of c-Myc through phosphorylation. 2) Reciprocal induction of c-Myc and the 4E-BP1 complex increases c-Myc expression: 4E-BP1 levels correlate with amplification of c-*Myc* in prostate cancer [34] and eIF4E activity increases c-Myc expression [35], while c-Myc overexpression also increases 4E-BP1 activity [34, 35] and induces translation initiation by the 4E-BP1 complex [36, 37]. 3) A feed-forward loop with c-Myc and the Tsc1/2 complex increases c-Myc: Loss of c-Myc increases *Tsc2* expression, which further represses c-Myc expression [26, 27], suggesting a functional c-Myc-Tsc2 loop. Thus, conversely, increased c-Myc with additional Tsc1 defects may completely relieve mTOR inhibition by the Tsc1/2 complex, inducing constitutive mTOR activation. In addition, a co-occurrence of c-*MYC* amplification and PI3K/mTOR pathway alteration has been observed in human prostate cancer, raising the possibility that these two genetic hits cooperate to promote tumor progression, and mouse models show that this cooperation accelerates progression of mPIN to microinvasive cancer [38]. Thus, it is important to determine whether there is a functional interaction of the Foxp3-c-Myc axis and Tsc1/2-mTOR pathway during tumor progression.

The mTORC1 inhibitor rapamycin reverses early mPIN lesions in young *Akt1* transgenic mice, but results were modest in *Pten* conditional knockout models [39, 40]. Moreover, recent clinical trials have shown that tumor resistance to rapamycin limits the efficacy of rapamycin or its analogues in inhibiting prostate cancer progression (ID: NCT00629525; http://www.clinicaltrials.gov). It has been proposed that these drugs do not completely block mTOR or other signaling pathways working in concert with the PI3K/mTOR pathway; the latter explanation has been supported by recent studies. Cells lacking c-Myc are sensitive to rapamycin, suggesting that some components of the mTOR pathway may be regulated by c-Myc [27]. Early studies suggested that c-Myc regulates the mTOR-targeting 4E-BP1 complex to control cell growth [36, 37], and recent studies revealed that c-Myc abrogates sensitivity to rapamycin through regulation of 4E-BP1 complex [34, 41], but its ability to confer rapamycin-resistance is not fully understood [34]. Furthermore, *in vivo* analysis showed that c-Myc expression can disrupt the elimination of mPIN lesions in young *Akt* mutant mice treated with mTOR inhibitors [38]. Thus, inhibition of c-Myc expression may be an effective means of alleviating resistance to mTOR inhibitors. In this proposed study, we will take advantage of our mouse models of prostate cancer to estimate the synergetic anti-tumor effects of co-treating mice with inhibitors of
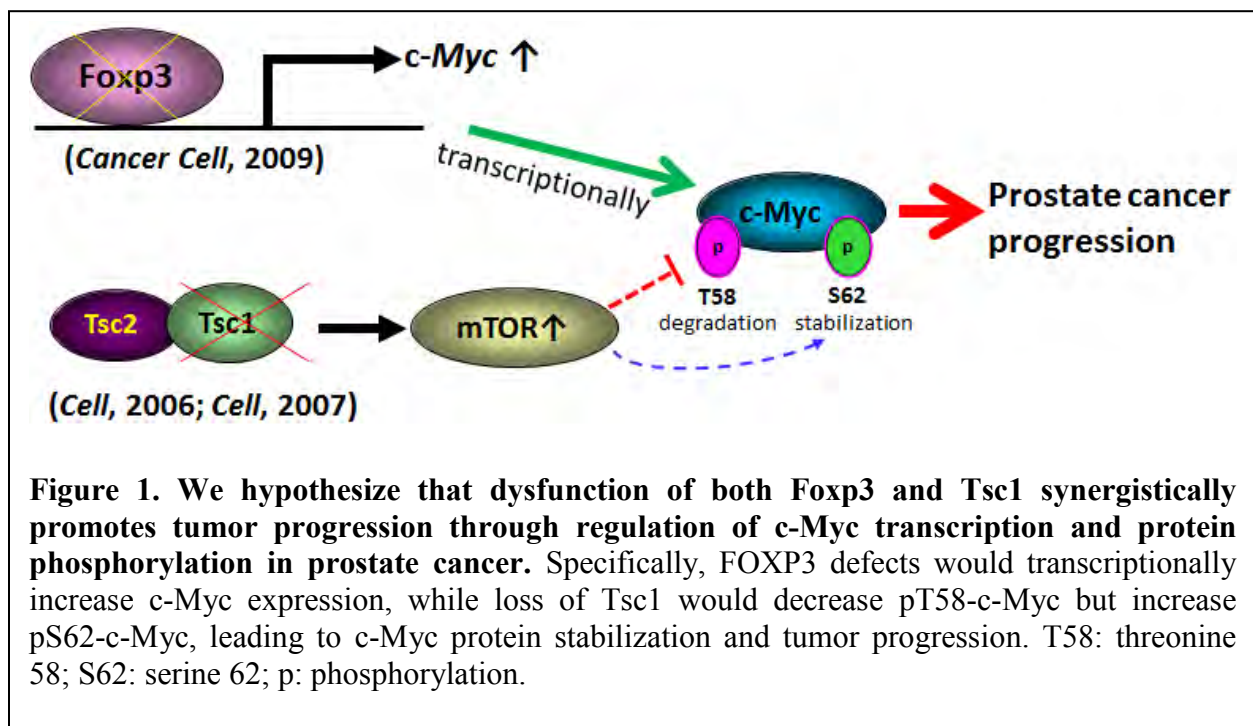
mTOR and c-MYC, a regimen that may prevent the development of tumor resistance to mTOR inhibitors.

KEYWORDS

Prostate cancer, Tumor progression, Gene therapy, Transcriptional regulation, Post-translational modification

ACCOMPLISHMENTS

Our preliminary studies provide evidence that the Foxp3-c-Myc axis and Tsc1-mTOR signaling pathways converge during tumor progression. First, lineage-specific ablation of *Foxp3* in mouse prostate leads to prostate hyperplasia and mPIN [8], but this does not occur when these mice are crossed with c-Myc-deficient mice, suggesting the Foxp3-c-Myc axis regulates tumor initiation. Second, introduction of a prostate-specific deletion of *Tsc1* into *Foxp3* mutant mice leads to prostate carcinoma at an early age, suggesting that the deletion of *Tsc1* accelerates *Foxp3*-related tumor progression. This phenotype was supported in human primary prostate cancers in which FOXP3 defects were often accompanied by deletions of *TSC1*. Third, Foxp3 can transcriptionally inhibit c-*Myc* expression [8] and mTOR can control c-Myc phosphorylation in prostate cancer cells. Since Tsc1 is an upstream inhibitor of mTOR [22, 23], c-Myc is likely to be a bridge for the cross-talk between Foxp3 and Tsc1 in tumor progression. Therefore, our central hypothesis is that dysfunction of both Foxp3 and Tsc1 synergistically promotes tumor progression through regulation of c-Myc transcription and phosphorylation/stabilization in prostate cancer (**Fig. 1**). Our overall goal is to define the mechanisms regulating the cross-talk between Foxp3 and Tsc1 in tumor progression and improve existing therapeutic strategies in prostate cancer.

Figure 1. We hypothesize that dysfunction of both Foxp3 and Tsc1 synergistically promotes tumor progression through regulation of c-Myc transcription and protein phosphorylation in prostate cancer. Specifically, FOXP3 defects would transcriptionally increase c-Myc expression, while loss of Tsc1 would decrease pT58-c-Myc but increase pS62-c-Myc, leading to c-Myc protein stabilization and tumor progression. T58: threonine 58; S62: serine 62; p: phosphorylation.

Task 1: IACUC approvals for mouse studies

The animal protocol has been approved by both the UAB IACUC and the USAMRMC Animal Care and Use Review Office (ACURO)
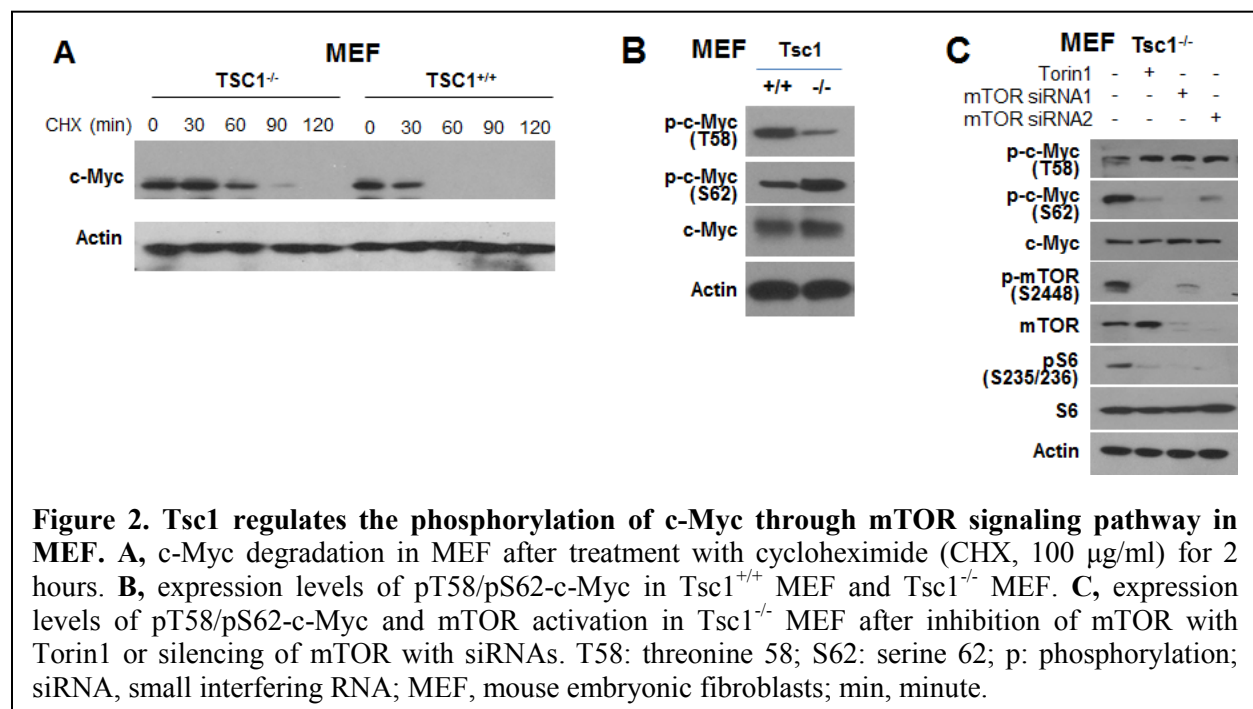
Specific Aim 1: To characterize the functional cross-talk between Foxp3 and Tsc1 in vitro

Task 2: Determine the cross-talk between Foxp3 and Tsc1 *in vitro*

Task 2a: The cross-talk among Tsc1, mTOR, c-Myc and Foxp3 in *Tsc1$^{-/-}$* and *Tsc1$^{+/+}$* MEF (2-12 months)

1. Tsc1-mTOR signaling modulates the phosphorylation and stabilization of c-Myc in MEF

To test if Tsc1 is implicated to c-Myc stability, we determined the half-life of c-Myc after treatment with the protein synthesis inhibitor cycloheximide (CHX) in mouse Tsc1$^{-/-}$ and Tsc1$^{+/+}$ MEF. While c-Myc was degraded after 30 min of treatment with CHX, the c-Myc degradation was more delayed in Tsc1$^{-/-}$ MEF than in Tsc1$^{+/+}$ MEF (**Fig. 2A**), indicating that *Tsc1*-deficiency stabilizes c-Myc in MEF. Furthermore, we observed that *Tsc1* knockout resulted in the simultaneous increase of pS62-c-Myc and decrease of pT58-c-Myc by a comparison of *Tsc1$^{-/-}$* and *Tsc1$^{+/+}$* MEF (**Fig. 2B**), suggesting that the phosphorylation state of c-Myc is affected by Tsc1-deficiency.

Figure 2. Tsc1 regulates the phosphorylation of c-Myc through mTOR signaling pathway in MEF. A, c-Myc degradation in MEF after treatment with cycloheximide (CHX, 100 μg/ml) for 2 hours. **B,** expression levels of pT58/pS62-c-Myc in Tsc1$^{+/+}$ MEF and Tsc1$^{-/-}$ MEF. **C,** expression levels of pT58/pS62-c-Myc and mTOR activation in Tsc1$^{-/-}$ MEF after inhibition of mTOR with Torin1 or silencing of mTOR with siRNAs. T58: threonine 58; S62: serine 62; p: phosphorylation; siRNA, small interfering RNA; MEF, mouse embryonic fibroblasts; min, minute.

Tsc1 is an essential component of the mTOR pathway and loss of Tsc1/2 facilitates mTOR activation [22, 23]. Thus, we investigated the effect of mTOR on expression of pT58/pS62-c-Myc in MEF. In *Tsc1$^{-/-}$* MEF, the simultaneous increase of pS62-c-Myc and decrease of pT58-c-Myc were observed after inhibition of mTOR with Torin1, a mTOR inhibitor of both mTORC1 and mTORC2 [32, 42, 43], or silencing of mTOR with siRNAs (**Fig. 2C**). The effective inhibition of mTOR activation was validated by a downregulation of p-mTOR and pS6. This data suggests a post-translational regulation of the phosphorylation and stabilization of c-Myc by Tsc1-mTOR signaling.

2. Synergistic cross-talk between Foxp3 and Tsc1 in the regulation of c-Myc in MEF

To determine the role of *Foxp3* in *Tsc1*-dependent regulation of pT58/pS62-c-Myc, we transfected *Foxp3* into both *Tsc1*$^{-/-}$ and *Tsc1*$^{+/+}$ MEF. The exogenous Foxp3 effectively blocked an increase of pS62-c-Myc by Tsc1-deficiency, while pT58-c-Myc appears to be lightly increased after *Foxp3* transfection in *Tsc1*$^{-/-}$ MEF. However, total c-Myc levels were dramatically reduced after *Foxp3* transfection in both *Tsc1*$^{-/-}$ and *Tsc1*$^{+/+}$ MEF (**Fig. 3A**). This data suggests a synergistic effect of *Foxp3* and *Tsc1* on c-Myc expression levels in MEF.

We observed that pT58/pS62-c-MYC is controlled by Tsc1-mTOR signaling (**Fig. 2B, C**), but it remains unknown whether this regulation is direct or indirect. Using siRNA, we identified that silencing of either mTOR or 4EBP1 attenuates the *Tsc1*-dependent regulation of pT58/pS62-c-Myc in MEF (**Fig. 2C and 3B**). However, it is still difficult to distinguish whether pT58/pS62-c-Myc levels are regulated by mTOR directly or through the 4E-BP1 complex, which can also induce c-Myc [34-37] and may, therefore, have a role in c-Myc phosphorylation and subsequent activity. In addition, Tsc2, Gsk3β, Pin1, PP2A, Cdk2, and MAPK [26, 27, 44-47] may also be implicated in the *Tsc1*-dependent regulation of c-Myc. However, we did not find any changes of these proteins in absence or present of Tsc1 and Foxp3 in MEF (**Fig. 3B**), supporting a direct regulation of c-Myc phosphorylation by mTOR or its substrates.
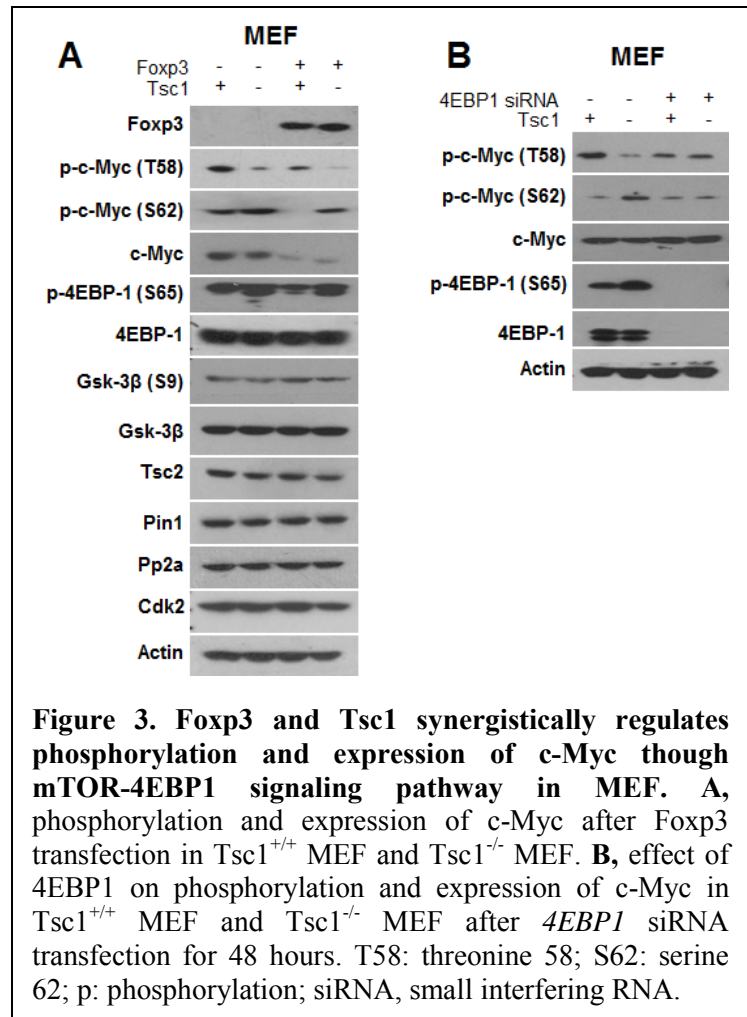
Figure 3. Foxp3 and Tsc1 synergistically regulates phosphorylation and expression of c-Myc though mTOR-4EBP1 signaling pathway in MEF. A, phosphorylation and expression of c-Myc after Foxp3 transfection in Tsc1$^{+/+}$ MEF and Tsc1$^{-/-}$ MEF. B, effect of 4EBP1 on phosphorylation and expression of c-Myc in Tsc1$^{+/+}$ MEF and Tsc1$^{-/-}$ MEF after *4EBP1* siRNA transfection for 48 hours. T58: threonine 58; S62: serine 62; p: phosphorylation; siRNA, small interfering RNA.

Task 2b: The cross-talk among FOXP3, TSC1, mTOR, and c-MYC in prostate cancer cells (2-9 months)

1. Functional cross-talk between FOXP3 and TSC1 in the regulation of c-Myc in prostate cancer cells

To confirm the involvement of TSC1 in regulating c-MYC in prostate cancer cells, we used siRNAs to silence human *TSC1* normally expressed in androgen-dependent prostate cancer cell line LNCaP. While total c-MYC levels were not changed, the simultaneous increase of pS62-c-Myc and decrease of pT58-c-Myc were observed after silencing of *TSC1* with siRNAs in LNCaP cells (**Fig. 4A**). The *TSC1*-dependent mTOR activation was also validated by increased expression levels of p-mTOR, p-4EBP1, and pS6. Next, we treated the androgen-independent prostate cancer cell line DU145 with mTOR inhibitors rapamycin and Torin1. Treatment for 2h

with high-dose rapamycin or Torin1 induced pT58-c-Myc and reduced pS62-c-Myc levels (**Fig. 4B**). This data suggests that inhibition of mTOR increases pT58-c-Myc and lightly decreases pS62-c-Myc but not at total c-MYC levels. This finding shows the first evidence that Tsc1-mTOR signaling regulates c-Myc phosphorylation in prostate cancer cells. Since *TSC1* is mutated and expressed only at low levels in DU145 cells, to validate the cross-talk between FOXP3 and TSC1 in prostate cancer cells, we will transfect WT *TSC1* and WT *FOXP3* into the DU145 cells and compare the levels of c-MYC and mTOR and their phosphorylation states. As shown in **Figure 4C**, an increase in pT58-c-Myc and a decrease in pS62-c-Myc were observed after *TSC1* transfection, but total c-MYC were reduced by transfections with both *TSC1* and *FOXP3*, suggesting that FOXP3 and TSC1 converge to inhibit c-MYC expression in prostate cancer cells. Thus, we provided a regulatory mechanism of the cross-talk between FOXP3 and
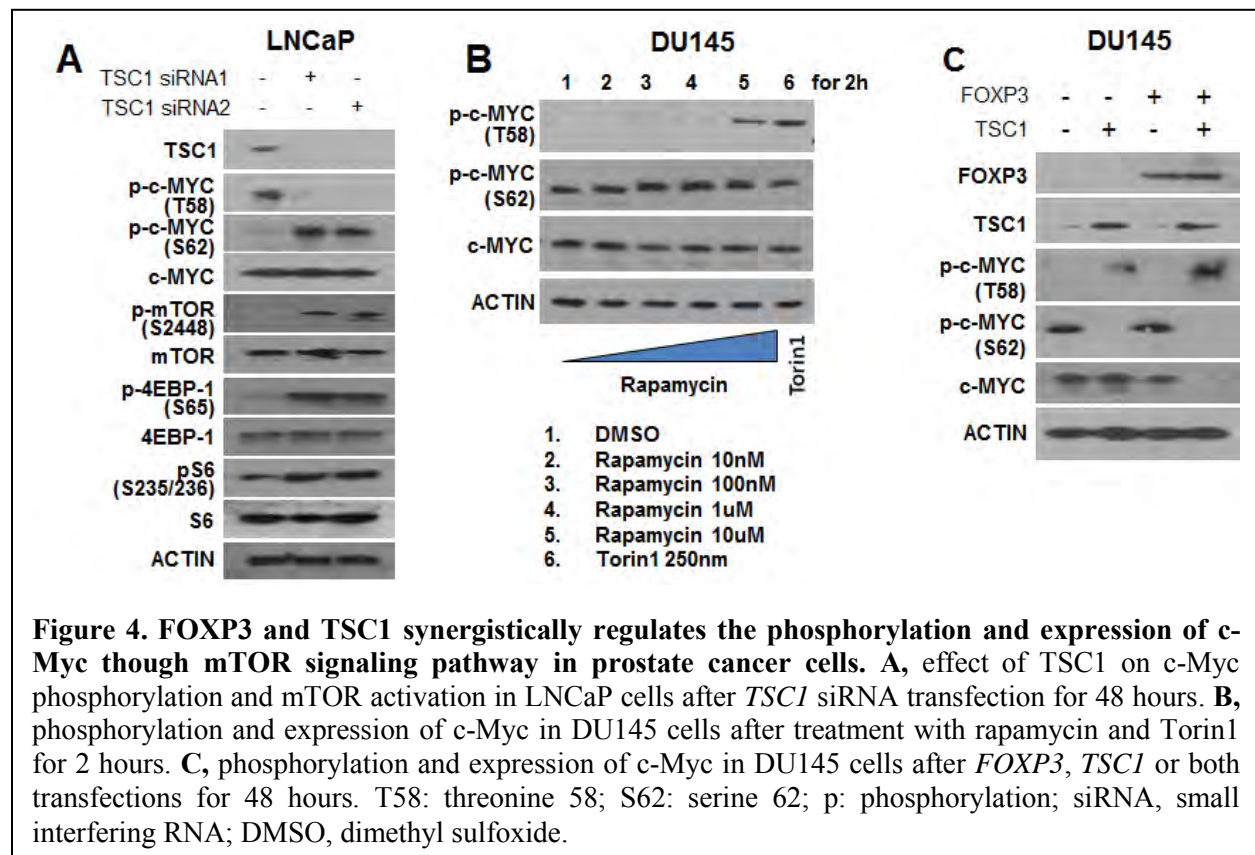

Figure 4. FOXP3 and TSC1 synergistically regulates the phosphorylation and expression of c-Myc though mTOR signaling pathway in prostate cancer cells. A, effect of TSC1 on c-Myc phosphorylation and mTOR activation in LNCaP cells after *TSC1* siRNA transfection for 48 hours. **B,** phosphorylation and expression of c-Myc in DU145 cells after treatment with rapamycin and Torin1 for 2 hours. **C,** phosphorylation and expression of c-Myc in DU145 cells after *FOXP3*, *TSC1* or both transfections for 48 hours. T58: threonine 58; S62: serine 62; p: phosphorylation; siRNA, small interfering RNA; DMSO, dimethyl sulfoxide.

TSC1 in regulation of c-Myc function in prostate cancer cells.

2. Dual targeting c-MYC and mTOR signaling pathways is an effective therapeutic approach for inhibition of prostate cancer cell growth

While the molecular mechanism of c-Myc-induced resistance to mTOR inhibition is still unknown, cells lacking c-Myc do not become resistant to mTOR inhibitors [41, 48]. Therefore, one of our aims is to determine if combination therapy with mTOR and c-Myc inhibitors can overcome such resistance. Recently, an *in vitro* analysis revealed that the phosphorylation sites of mTORC1 substrates correlate strongly with the resistance to rapamycin [49]. Thus, the intrinsic capacity of a phosphorylation site to serve as an mTORC1 substrate is a major determinant of its sensitivity to mTOR inhibitors. Our data shows that c-Myc phosphorylation at T58 is regulated by high-dose rapamycin or Torin1 but is resistant to normal-dose rapamycin

(**Fig. 4B**), suggesting that this c-Myc phosphorylation site may be associated with resistance to rapamycin.

We tested the synergistic action by blocking mTOR and c-Myc activity with inhibitors of both proteins in three commonly used prostate cancer cell lines: DU145, PC3, and LNCaP (**Fig. 5**). The mTOR inhibitors included rapamycin, Torin1, INK128, and DG-2; the c-Myc inhibitors included JQ1, 10058-F4, and 10074-G5. To observe the synergistic action of mTOR and c-Myc inhibitors, we administered only half of the effective dosage of each drug to the prostate cancer cells. Several combinations of these inhibitors reduced cell growth, but the combination of Torin1 and 10058-F4 inhibited cell growth most substantially in all three cell lines (**Fig. 5**).

Figure 5. Effects of mTOR and c-Myc inhibitors on prostate cancer cell growth. Cells were treated with half of the effective dosage of each drug. Error bars represent standard deviation. Rap: rapamycin; F4: 10058-F4; DMSO: dimethyl sulfoxide.
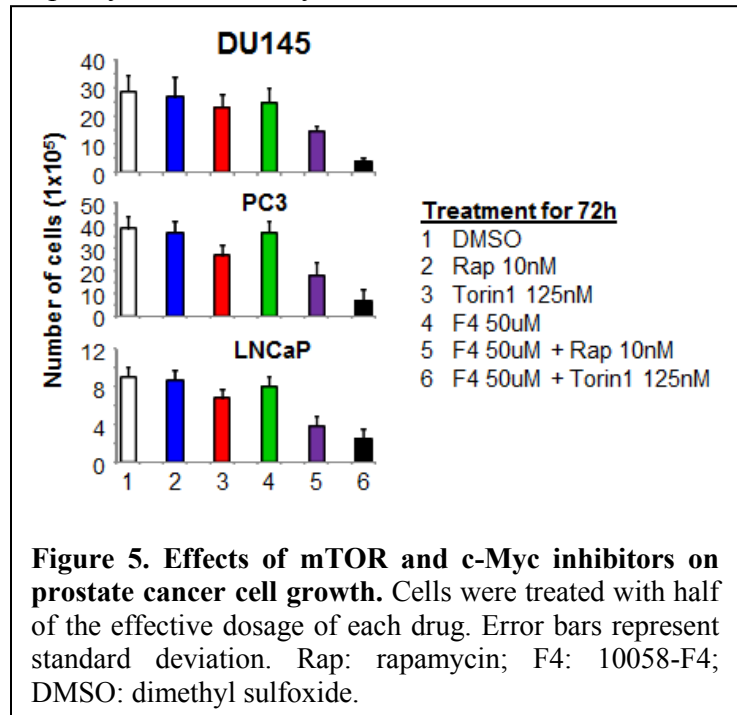
Torin1 is an ATP-competitive mTOR inhibitor that directly inhibits both mTORC1 and mTORC2 and impairs cell growth and proliferation to a far greater degree than rapamycin [32, 42, 43, 50, 51]. At 250 nM, Torin1 completely inhibited proliferation and caused a G1/S cell cycle arrest in MEFs [51]. 10058-F4 is a c-Myc inhibitor that prevents the binding of c-Myc/Max dimers to DNA targets, downregulates c-Myc expression, abrogates various c-Myc–dependent functions, and induces cell-cycle arrest and apoptosis [52-55]. Treatment with 100 μM 10058-F4 over 72 h effectively inhibited cell growth in AML cells [52]. Thus, dual targeting c-MYC and mTOR signaling pathways by a combination of 10058-F4 and Torin1 is an effective therapeutic approach for inhibition of prostate cancer cell growth.

Specific Aim II: To validate the functional cross-talk between Foxp3 and Tsc1 *in vivo*

Task 3: Validate the cross-talk between Foxp3 and Tsc1 *in vivo*

Task 3a: The c-Myc expression and phosphorylation in the *Tsc1* and/or *Foxp3* knockout prostates (2-12 months)

1. Prostate-specific deletion of *Tsc1* accelerates prostate tumor progression in *Foxp3* mutant mice

We introduced a prostate-specific deletion of the potential tumor suppressor gene *Tsc1* into *Foxp3* mutant mice on a C57BL/6 background. *Tsc1*/*Foxp3* double-deletions in the mouse prostate led to tumor progression starting at 22 weeks of age, and 100% of these mice developed prostate carcinoma by 43 weeks of age (**Fig. 6A-D**). At high magnification, definitive alterations in nuclear and cytoplasmic features are evident in prostate tissue, including larger and more vesicular nuclei and more densely eosinophilic cytoplasm. Similar cytologic alterations are well characterized in a variety of early invasive carcinomas in humans [56]. Interestingly, these

10

features are reminiscent of high-grade PIN and associated invasive carcinoma (Gleason pattern 3) in human prostate cancer. In immunohistochemistry (IHC) analysis, increased Ki67$^+$ cells and

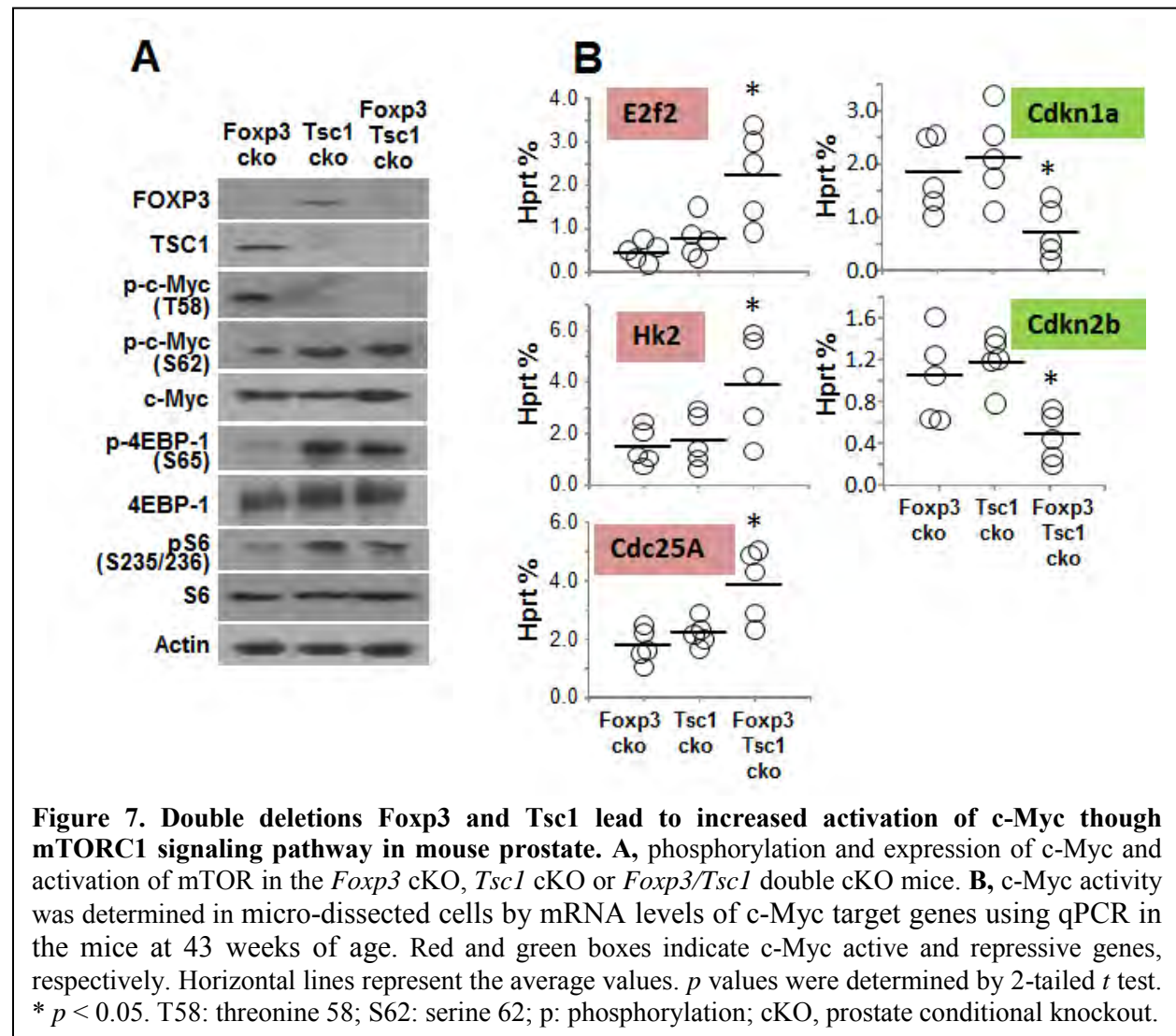**Figure 6. Prostate-specific double-deletions of *Tsc1* and *Foxp3* promote tumor progression. A,** tumor growth in the lateral lobe of the prostate in a 22-week-old *PB-Cre4$^+$Foxp3$^{f/y}$ Tsc1$^{f/f}$* C57BL/6 (double-mutant) mouse, identified by H&E staining. The high-power magnification in the right panel shows atypical epithelial cells with enlarged nuclei characteristic of prostate adenocarcinoma. Blue arrows indicate potential microinvasion. **B,** double-deletions of *Tsc1* and *Foxp3* led to increased Ki67$^+$ but decreased E-cadherin$^+$ prostate cancer cells compared with adjacent normal prostate epithelial cells, using IHC analysis of Ki67 and E-cadherin as indicators of proliferating cells and potential metastatic cells. **C,** survival analysis for prostate cancer incidence among *Foxp3* (*PB-Cre4$^+$Foxp3$^{f/y}$*), *Tsc1* (*PB-Cre4$^+$Tsc1$^{f/f}$*), and *Foxp3*/*Tsc1* double-mutant (*PB-Cre4$^+$Foxp3$^{f/y}$ Tsc1$^{f/f}$*) mice (n = 40/group). At 7, 12, 17, 22, 27, 32, 37, and 43 weeks, 5 mice per group were sacrificed for pathologic analysis. Within the 43-week observation period, only *Foxp3*/*Tsc1* double-mutant mice developed prostate carcinoma after 22 weeks of age. **D,** tumor development and progression in single- and double-mutant mice. Blue arrows indicate the time of mPIN development; red arrows indicate the time of cancer development.

11

decreased E-cadherin^{+} cells were observed in the cancer lesions compared with adjacent normal tissues (**Fig. 6B**), indicating high proliferative and potential metastatic tumor cells. In survival analysis, only double deletions of *Tsc1* and *Foxp3* leads to prostate carcinoma at an early age (**Fig. 6C**). Both *Foxp3* single-mutant mice and *Tsc1* single-mutant mice develop mPIN within one year [8, 24], but *Foxp3/Tsc1* double-mutant mice develop prostate cancer much earlier at 22 to 43 weeks of age (**Fig. 6D**), suggesting loss of both genes hastens tumor progression. These results suggest that *Tsc1/Foxp3* double-deletions facilitate malignant transformation, but the mechanism by which the interaction of *Foxp3* and *Tsc1* controls tumor progression remains to be clarified.

2. Double deletion of *Tsc1* and/or *Foxp3* changes c-Myc phosphorylation, expression and activation in mouse prostates

     Foxp3 deletion in the prostate increases c-*Myc* levels [8] and *Tsc1* deletion in the prostate increases mTOR activation [24], which may stabilize c-Myc protein by regulating its phosphorylation at T58 and S62 in prostate cancer cells (**Fig. 4**). Thus, c-Myc may be a bridge for the functional cross-talk between *Foxp3* and *Tsc1* in the double-mutant mice. We determined the levels of total c-Myc and pS62/pT58-c-Myc protein in prostates of *Foxp3*, *Tsc1*, and

Figure 7. Double deletions Foxp3 and Tsc1 lead to increased activation of c-Myc though mTORC1 signaling pathway in mouse prostate. A, phosphorylation and expression of c-Myc and activation of mTOR in the *Foxp3* cKO, *Tsc1* cKO or *Foxp3/Tsc1* double cKO mice. **B,** c-Myc activity was determined in micro-dissected cells by mRNA levels of c-Myc target genes using qPCR in the mice at 43 weeks of age. Red and green boxes indicate c-Myc active and repressive genes, respectively. Horizontal lines represent the average values. *p* values were determined by 2-tailed *t* test. * *p* < 0.05. T58: threonine 58; S62: serine 62; p: phosphorylation; cKO, prostate conditional knockout.

Foxp3/Tsc1 double-mutant mice at 43 weeks of age by immunoblotting with specific antibodies. The levels of c-Myc protein were higher in the *Foxp3/Tsc1* double-mutant mice than in the single-mutant mice (**Fig. 7A**), suggesting that the tumor progression is mediated by a synergistic upregulation of c-Myc. Likewise, expression levels of p4E-BP1 and pS6 were also be assessed in these mice and an upregulation of mTOR activation were also observed in *Tsc1* and *Foxp3/Tsc1* double-mutant mice. Furthermore, we obtained normal and cancer epithelial cells by a laser capture microdissection from mouse prostate and tumor tissues. The c-Myc activity was evaluated in the micro-dissected cells by measuring the mRNA expression levels of c-Myc target genes, such as *E2f2*, *Hk2*, *Cdc25A, Cdkn1a* and *Cdkn2b*, using quantitative PCR (qPCR). As shown in **Figure 7B**, the expression levels of c-Myc target genes is greater in activated genes *E2f2*, *Hk2* and *Cdc25A* but lower in repressed genes *Cdkn1a* and *Cdkn2b* in the cancer cells from double-mutant mice than in the mPIN cells from single-mutant mice, further suggesting that tumor progression in the double-mutant mice is caused by an elevated c-Myc function.

Task 3b: The synergistic action of Foxp3 and c-Myc phosphorylation in tumor progression (2-30 months).

This task still on going for observation in targeting mouse models, prostate c-Myc^{WT} and c-Myc^{T58A} knock-in mice, by ex vivo imaging (MIR, Medical Imaging Resources).

IMPACT

We have validated our hypothesis *in vitro* and part of *in vivo* and identify of a novel genetic mechanism in the pathogenesis of prostate cancer progression.

1. Understanding the pathogenesis of prostate cancer progression

While c-Myc and mTOR are the most frequently activated proteins in prostate cancer, their interaction is largely untested. Ours is the first attempt to investigate the synergy between the Foxp3 and Tsc1 pathways during tumor progression. In human prostate cancer, FOXP3 defects are often accompanied by deletions of *TSC1*. Prostate-specific deletions of *Foxp3* and *Tsc1* in mice led to tumor progression at an early age. c-Myc is one of the most commonly overexpressed oncogenes in prostate cancer. However, the mechanisms regulating c-Myc function in prostate cancer remain largely unknown. Foxp3 inhibits c-*Myc* transcription, and our data in this proposed study suggest that mTOR signaling affects phosphorylation of c-Myc in prostate cancer cells. The relative levels of phosphorylation at specific positions within c-Myc determine its protein stability. In this proposed work, we identified the cross-talk between Foxp3 and Tsc1 that have a critical role in c-Myc expression and stability in prostate cancer cells and mouse prostate cancers. This mechanism will help to understand why double Foxp3 and Tsc1 deficiencies promote tumor progression of prostate cancer as well as to understand how c-Myc is activated and its role in tumor progression of prostate cancer.

2. Understanding the mechanism of resistance to mTOR inhibitors

The mTOR inhibitor rapamycin and its analogs have been used in clinical trials. Unfortunately, cancer cells eventually develop a resistance to rapamycin, which may be due to ineffective block of mTOR or other signaling pathways working in concert with the mTOR pathway. The latter explanation has been supported by recent studies. Cells lacking c-Myc are sensitive to the effects of rapamycin, but in mice, tumors overexpressing c-Myc are resistant to rapamycin, suggesting that c-Myc is involved in rapamycin-resistance. Since both mTOR and c-Myc are frequently activated in prostate cancer, dual targeting strategies may be an effective

approach for prostate cancer therapy. In this proposed work, we tested a joint administration of rapamycin or a novel mTOR inhibitor (e.g., Torin1) with a c-Myc inhibitor (e.g., 10058-F4) in prostate cancer cells. While rapamycin was ineffective in the inhibition of cancer cell proliferation, a combination of rapamycin with 10058-F4, especially Torin1 with 10058-F4 can overcome this resistance. This identification provides a promising therapeutic approach against prostate tumor progression for patients with resistance to mTOR inhibitors.

CHANGES/PROBLEMS

None

PRODUCTS

1. Liu R, Liu C, Chen D, Yang WH, Liu X, Liu CG, Dugas CM, Tang F, Zheng P, Liu Y and **Wang L***. FOXP3 controls an miR-146/NFκB negative feedback loop that inhibits apoptosis in breast cancer cells. *Cancer Research*, 2015 Apr 15; 75(8):1703-13. PMID: 25712342.

2. Liu R, Yi B, Wei S, Yang WH, Har KM, Chauhan P, Zhang W, Mao X, Liu X, Liu CG and **Wang L***. FOXP3-microRNA-146-NF-κB axis and therapy for precancerous lesions in prostate. *Cancer Research*, 2015 Apr 15;75(8):1714-24. PMID: 25712341.

3. Meredith LJ, Wang CM, Nascimento L, Liu R, **Wang L**, Yang WH. The Key Regulator for Language and Speech Development, FOXP2, is a Novel Substrate for SUMOylation. *J Cell Biochem*. 2015, in press. PMID: 26212494.

4. Etikala DM, Liu R, **Wang L***. FOXP3-microRNA-146-NF-κB as oncotarget. *Oncoscience*, 2015, in press.

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SPECIAL REPORTING REQUIREMENTS

None

APPENDICES

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